

**Disruption of termite gut-microbiota and its  
prolonged fitness consequences<sup>†</sup>**

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Keywords: colony fitness, gut microbiota, mutualism, oogenesis, social  
insects

Running title: Fitness costs in antibiotic-treated termites

<sup>†</sup>Supplemental material for this article may be found at <http://>

**ABSTRACT**

26 The disruption of host-symbiont interactions through the use of antibiotics  
can help elucidate microbial functions that go beyond short-term nutritional  
28 value. Termite gut symbionts have been studied extensively, but little is  
known about their impact on the termite's reproductive output. Here we  
30 describe the effect that the antibiotic rifampin has not only on the gut  
microbial diversity, but also on the longevity, fecundity, and weight of two  
32 termite species - *Zootermopsis angusticollis* and *Reticulitermes flavipes*.  
We report three key findings: (i) the antibiotic rifampin, when fed to  
34 primary reproductives during the incipient stages of colony foundation,  
causes a permanent reduction in the diversity of gut bacteria, and a  
36 transitory effect on the density of the protozoan community, (ii) rifampin  
treatment reduces oviposition rates of queens, translating into delayed  
38 colony growth and ultimately reduced colony fitness and (iii) the initial  
dosages of rifampin on reproduction and colony fitness had severe long-  
40 term fitness effects on *Z. angusticollis* survivorship and colony size. Taken  
together, our findings demonstrate that the antibiotic-induced perturbation  
42 of the microbial community associates with prolonged reductions in  
longevity and fecundity. A causal relationship between these changes in the  
44 gut microbial population structures and fitness is suggested by the  
acquisition of opportunistic pathogens and incompetence of the termites to  
46 restore a pre-treatment, native microbiota. Our results indicate that  
antibiotic treatment significantly alters the termite's microbiota,

48 reproduction, colony establishment and ultimately, colony growth and  
development. We discuss the implications for antimicrobials as a new  
50 application to the control of termite pest species.

## INTRODUCTION

52                   The long-standing associations between termites and their  
prokaryotic and eukaryotic microorganisms have been crucial to the  
54                   evolutionary and ecological success of this social insect group. The  
presence of cellulolytic microorganisms in the hindguts of termites is one  
56                   of the key events that allowed termites to thrive on nitrogenous deficient  
food resources (49, 63). Fossil records (80) and the similarity in gut flora  
58                   and other microbial endosymbionts with those of their roach relatives (59)  
support the hypothesis that these associations existed in the termite  
60                   ancestor (3, 50, 59). Termite gut symbionts reside in the lumen or are  
attached to the wall of the hindgut region and can represent more than 40%  
62                   of the termite's weight (6). They are horizontally transmitted through  
coprophagy, a common behavior in termites. Indeed, the need for  
64                   transfaunation of hindgut symbionts has been proposed as one of the main  
factors favoring group-living (44) and specifically, favoring the evolution  
66                   and maintenance of termite sociality (18). Yet, little is known about the  
impact that termite gut symbionts have beyond their role in cellulose  
68                   degradation and host nutrition.

                  Here we report on the impact that antibiotic treatment has on the  
70                   reproductive survival and fecundity of the dampwood termite *Zootermopsis*  
*angusticollis* and the Eastern subterranean termite *Reticulitermes flavipes*.  
72                   Although previous experiments demonstrated that antibiotics compromise  
and/or eradicate the gut microbiota (protozoa and/or bacteria) of termites

74 (11, 26, 53), no studies have yet characterized the short- and long-term  
fitness costs associated with antibiotics in these social insects, nor their  
76 impact on colony growth and development. Our findings suggest that  
rifampin disrupts one or more mutualistic interactions essential for normal  
78 termite reproduction and longevity.

## 80 MATERIALS AND METHODS

**Collection and maintenance of termites.** Two mature colonies of *Z.*  
82 *angusticollis* were collected from Huddart Park, San Mateo, CA. and  
maintained as described in Rosengaus (57). Reproductives of *R. flavipes*,  
84 an important structural pest in the USA, were collected from two stock  
colonies from West Roxbury, Massachusetts.

86  
**Establishment of incipient colonies.** Incipient colonies were bred in the  
88 laboratory from virgin alates (winged adult dispersal forms). These fully  
pigmented individuals were collected, sexed and paired only if their wings  
90 could be removed easily when folded anteriorly along the humeral suture  
(57). These selection criteria guaranteed that only ready to disperse virgin  
92 females and males were used in our studies. To prevent mating prior to  
colony establishment, the de-winged reproductives were housed in same  
94 sex/same colony containers (18 x12 x 8 cm) lined with moist paper towels  
and some nest material. Within seven days of removal from the parental  
96 nest, reproductives pairs were placed inside Petri dishes (60 x 15 mm) lined

with filter paper (Whatman qualitative #1) and approximately 5.0 g of  
98 decayed birch wood. Subsequently, the filter paper was moistened with  
either distilled water (controls) or rifampin (Sandoz Inc, Princeton, NJ; 300  
100 mg capsules; see below for details). Rifampin is bacteriostatic or  
bactericidal depending on dosage and acts by specifically inhibiting DNA-  
102 dependent RNA polymerase activity in Eubacterial cells (27). It is a broad-  
spectrum compound active against a variety of gram-positive and gram-  
104 negative organisms (8, 16, 55, 76). The dishes, stacked in covered plastic  
boxes (30 x 23 x 10 cm), were maintained at 22°C.

106

**Effects of antibiotic ingestion on *Z. angusticollis* gut microbiota.** To  
108 determine if rifampin affected the composition of the termite's gut  
microbial community, *Z. angusticollis* reproductive pairs were established  
110 in incipient colonies as described earlier. The diet of four incipient colonies  
was supplemented with 300 µL of a 0.5% suspension of rifampin on the  
112 day of pairing and 14 and 34 days after the initial dose. Three  
corresponding control colonies were similarly established but received  
114 distilled water instead. Subsequently, these colonies were left undisturbed  
until day 85 post-pairing when control and rifampin-fed females were  
116 surface sterilized with 2% NaClO and then their guts dissected in sterile  
PBS buffer and preserved in 70% molecular grade ethanol. This time frame  
118 was chosen because it was approximately at this time that the initial  
differences in oviposition rates became evident. Each sample was then  
120 centrifuged at 12,000X G, and the ethanol was decanted. The DNA of the

guts was extracted using the QUIAGEN DNeasy Blood & Tissue kit per  
122 the manufacturer's instructions for "Purification of Total DNA from  
Animal Tissues". All samples were homogenized and treated with  
124 proteinase K for 3 hr at 55°C before the column extraction procedure.  
Aliquots of the resulting DNA samples were then pooled and stored at 4°C  
126 until PCR, cloning and sequencing (see Supplemental material for detailed  
protocol). Extraction controls of sterile water were treated identically to  
128 samples and carried through all subsequent procedures. Negative water  
controls, as expected, showed no PCR amplification and did not yield  
130 clones containing an insert.

132 **Effects of antibiotic ingestion by *Z. angusticollis* on abundance of**  
**eukaryotic microbes.** To assess the effect of rifampin on the abundance of  
134 eukaryotic symbionts, we quantified protozoa in the guts of *Z. angusticollis*  
nymphs (given the unavailability of reproductives since they are produced  
136 only once a year). To control for the possible effect of termite density on  
gut symbionts and simulate social conditions between the two  
138 reproductives, pairs of control ( $N = 26$ ) and 0.5% rifampin-fed ( $N = 30$ )  
nymphs were established and hindgut protozoa density was estimated on  
140 the third, eighth and 14<sup>th</sup> day post-treatment. These nymphs were first  
surface sterilized by submersion in 5% hypochlorite solution for 60  
142 seconds followed by two consecutive one-minute washes in sterile water.  
Subsequently, their entire gut was dissected. The gut, placed inside a 1.5  
144 mL sterile microcentrifuge tube containing 1000  $\mu$ L of U solution (73), was



homogenized with a sterile pestle. To quantify the protists, 10  $\mu$ L of the  
146 suspension was immediately transferred to a hemocytometer and the  
number of intact and active protozoa was recorded. These estimates likely  
148 represent an underestimate of the total eukaryotic microbial community as  
the possibility of lysis of the anaerobic protozoa existed during this  
150 procedure. Given that both the control and experimental animals were  
treated in an identical manner, our quantification allows for a relative  
152 measure of the impact that rifampin had on gut protozoa between the  
treatments rather than providing an absolute density of such microbes.

154

**Survival of *Z. angusticollis* and *R. flavipes* reproductives and colony**  
156 **fitness.** Incipient colonies were established as described above to examine  
the effect of rifampin on termite survival and fitness. These colonies  
158 ensured the monitoring of complete families throughout colony ontogeny  
by performing periodic censuses. The filter paper was initially moistened  
160 with either 300  $\mu$ L of distilled water (controls) or 300  $\mu$ L of a 0.5%  
rifampin solution (dissolved in sterile water) on the day of pairing and then  
162 again on the third (50  $\mu$ L of water or rifampin) and seventh day post-  
establishment (100  $\mu$ L of distilled water or rifampin). Hence, the filter  
164 paper upon which experimental termites fed was impregnated with a total  
of 2.2 mg of rifampin throughout the entire length of the experiment. *Z.*  
166 *angusticollis* colonies were followed throughout the first 730 days post-  
pairing while the survival and fitness parameters for *R. flavipes* colonies  
168 were monitored for 150 days post-pairing. For *Z. angusticollis*, a total of 87

( $N = 49$  control and 38 experimental replicates) and 132 ( $N = 65$  control  
170 and 67 experimental replicates) nestmate pairs were initially established  
from each of the two stock colonies, BDTK19 and BDTK17, respectively.  
172 In addition, 29 incipient colonies were established by pairing non-nestmate  
male and female reproductives from these same two stock colonies (i.e.  
174 non-sibling pairs,  $N = 14$  control and 15 experimental replicates). For *R.*  
*flavipes*, control ( $N = 49$ ) and experimental nestmate pairs ( $N = 49$ ) were  
176 treated in an identical manner as the *Z. angusticollis* incipient colonies.

Incipient colonies of *Z. angusticollis* and *R. flavipes* underwent  
178 censuses every third day for the first 50 days post-pairing. During these  
frequent initial censuses, when the incipient colonies were housed in Petri  
180 dishes (Fig. 1), we recorded survival of the reproductives, the time elapsed  
till first oviposition and first hatching. Subsequently, colonies were  
182 censused approximately on day 150 following initial pairing for both  
termite species. For *Z. angusticollis*, the entire colony was then transferred  
184 to a larger covered plastic container (15 x 10 x 6 cm) lined with moist  
paper towels and decayed birch (~12 x 6 x 6 cm wood block) to allow  
186 colony expansion. They were left undisturbed until the 465 and 730 day  
census except for the addition of wood and water when needed (Fig. 1).

188  
**Effect of antibiotic ingestion on termite mass.** To test if rifampin  
190 supplementation during the initial stages of *Z. angusticollis* colony  
foundation negatively impacted the reproductive's nutritional health and  
192 thus, their survival and fitness, we recorded on day 50 and 465 post-

194 establishment the mass of each surviving reproductive as an indirect  
measure of nutritional status. For *R. flavipes*, the mass of the surviving  
196 reproductives was determined immediately after the 150 day census. No  
differences in the rates of wood and filter paper consumption were  
observed between control and antibiotic-fed reproductives for either  
198 species.

## 200 RESULTS

**Effects of antibiotic ingestion on *Z. angusticollis* gut microbiota.** Diets  
202 of *Z. angusticollis* reproductives were supplemented with a low dose of  
rifampin antibiotic suspension (0.005 grams of rifampin in 1 ml of sterile  
204 deionized water) on days 0, 14, and 34 after pairing. Sampling of the gut  
bacterial diversity by cloning and sequencing of 16S rRNA gene amplicons  
206 at day 85 indicated there was a significant difference in the bacterial  
population structures between the control and rifampin-treated termites of  
208 *Z. angusticollis* ( $P = 0.01$ , UniFrac; Table 1). As expected, rifampin  
treatment reduced the 16S rRNA gene bacterial diversity (Table 1). Of the  
210 87 clones sequenced from the control termite 16S rRNA gene library, 17  
operational taxonomic units (OTUs) were represented based on a 97%  
212 identity cutoff (mean Chao1 =  $23 \pm 6$  OTUs, mean ACE = 21). However,  
among the 85 clone sequences in the rifampin-treated termites, only six  
214 OTUs were represented (mean Chao1 =  $6 \pm 1$  OTUs, mean ACE = 6),  
amounting to a 64% reduction in bacterial diversity. The rarefaction

216 analyses of the two libraries also showed that despite similar sequencing  
efforts in each treatment, the control termite library was less exhaustively  
218 sampled than the antibiotic-treated termites (Fig. S1 Supplemental Material),  
indicating a greater diversity in the untreated termites. The species richness  
220 and diversity indices confirmed that there was an unequal distribution of  
the bacterial OTUs in both treatments (reciprocal Simpson's evenness = 6,  
222 control; 3, rifampin).

Of the six OTUs in the rifampin-treated guts, three were shared  
224 with the control group and two of these were maintained at the same  
relative proportion among the treatments (Table 1). These three bacteria  
226 include a *Treponema* sp.; the endomicrobia termite symbiont, Termite  
Group 1; and a *Desulfovibrio* sp. These bacteria are known inhabitants of  
228 termite guts (35, 40, 48). The other three OTUs in the treated group were  
unique and included *Serratia*, an uncultured *Enterococcus*, and an  
230 uncultured Epsilonproteobacterium that was the dominant bacteria in the  
rifampin-treated guts (Table 1). Given that resistance to rifampicin is easily  
232 attained by single random mutations of the bacterial RNA polymerase (16),  
it was necessary to establish whether the recorded alterations in gut  
234 microbial communities were influenced by a build-up of antibiotic-resistant  
species. After cross referencing our microbial diversity data with the  
236 expected species that contain these resistance mutations (Table S1  
Supplemental Material), we found that the frequency of strains with  
238 rifampin resistance was low and equivalent between pre- and post-

treatments ( $P = 0.6$ , Fisher's exact test). Thus, we conclude that the  
240 antibiotic treatment did not select for rifampin resistance.

Ingestion of rifampin also had a significant short-term negative  
242 impact on the number of gut protozoa per gram of termite. In a separate  
experiment, nymphs fed rifampin for three days had a significantly lower  
244 median number of protozoa ( $\pm$  interquartile range) in their gut relative to  
the controls (median rifampin =  $9.7 \times 10^6 \pm 3.4 \times 10^6$  vs. median control =  $2$   
246  $\times 10^7 \pm 9.4 \times 10^6$ ,  $P = 0.01$ ; medians are reported given that the frequency  
with which gut protozoa were recorded was not normally distributed).  
248 However, in subsequent dissections on days eight and 14 post-feeding, the  
number of protozoa per gram of termite did not differ significantly between  
250 the two treatments (median control =  $1.2 \times 10^7 + 9.3 \times 10^6$  vs. median  
rifampin =  $8.7 \times 10^6 + 7.6 \times 10^6$ ,  $P = 0.5$  on day eight; median control =  $9.8 \times$   
252  $10^6 \pm 1 \times 10^7$  vs. median rifampin =  $7 \times 10^6 \pm 9.3 \times 10^6$ ;  $P = 0.5$  on day 14).  
Thus, although rifampin temporarily affected the number of protozoa in  
254 termite guts, it did not destroy them completely. Collectively, our results  
indicate that rifampin has only a moderate and transitory effect on the  
256 density of the culturable, protozoa gut community, and a prolonged effect  
on the diversity of bacteria in termite guts.

258

**Survival of *Z. angusticollis* reproductives and colony fitness.** The effects  
260 of the antibiotic on survival was evaluated throughout the first two years of  
colony life. A Cox proportional regression model with the variables  
262 "colony of origin" (either BDTK17 or BDTK19), "gender", "sibship"

(nestmate or non-nestmate pairs) and “treatment” (controls or antibiotic-fed) revealed that colony of origin ( $WS = 7.3$ ,  $d.f. = 1$ ,  $P = 0.007$ ) and treatment ( $WS = 25.1$ ,  $d.f. = 1$ ,  $P < 0.0001$ ) had significant effects. First, reproductives from colony BDTK17 had 1.3 times the hazard ratio of death in comparison to reproductives from colony BDTK19, after controlling for the effect of treatment (Table 2). Second, rifampin-fed reproductives after two years post-pairing were 1.7 times as likely to suffer premature mortality compared to untreated individuals, even after controlling for the effect of colony of origin (Fig. 2; Table 2).

The time course of survival for the reproductives did not differ significantly between the rifampin and control treatments (Breslow  $X^2 = 2.8$ ,  $d.f. = 1$ ,  $P = 0.09$  for BDTK17 and Breslow  $X^2 = 0.1$ ,  $d.f. = 1$ ,  $P = 0.7$  for BDTK19; Fig. 2), until after day 150 (Fig. 2). By 465 days, the survival distributions and percent survival were significantly different between the control and antibiotic treatments for each of the stock colonies (Fig. 3). These differences were pronounced by 730 days. At this time, 50% of the original control reproductives had died. In contrast, the rifampin-fed reproductives reached 50% mortality by the 465 day census (Fig. 2; Table 2). Thus, on average, the control termites lived approximately 265 additional days before reaching the 50% mortality mark ( $LT_{50}$  estimate, Table 2). These findings indicate that the effects of rifampin treatment significantly affect survivorship of reproductives from both stock colonies, with mortality differences being most prominent between 465 and 730 days (Fig. 2; Table 2).

288 Rifampin-fed reproductives originating from both colonies had  
consistently fewer offspring than their corresponding untreated controls.

290 Because none of the reproductive output metrics differed significantly  
between BDTK17 and BDTK19 (Mann-Whitney U tests), statistical  
292 analyses were carried out by combining all colonies within a treatment.  
Given the longitudinal nature of this study, we present a detailed  
294 description of the effects of antibiotic treatment on colony fitness at each of  
the census dates.

296 *150 days post-pairing census.* The addition of low dosages of  
rifampin during the initial stages of colony foundation in *Z. angusticollis*  
298 resulted in a significant reduction in fecundity. Fig. 3 shows a significant  
disparity between the frequency distribution of offspring number between  
300 surviving control and rifampin-treated colonies. The percentage of  
surviving control colonies with eggs, larvae and soldiers on day 150 post-  
302 establishment was higher than that of rifampin-treated colonies;  
furthermore, a higher percentage of control colonies produced the highest  
304 number of eggs, larvae and soldiers (Fig. 3). One hundred fifty days post-  
pairing, surviving control colonies also had a significantly higher median  
306 number of eggs, larvae and soldiers than their rifampin counterparts (Fig.  
4). Furthermore, the effect of the antibiotic on *Z. angusticollis* reproductive  
308 output appeared to be immediate, since it significantly delayed first  
oviposition by approximately 47 days (MW = 870,  $z = -5.5$ ,  $P < 0.0001$ ;

310 Fig. 5a) and had a tendency to delay first hatching by roughly 33 days  
 (MW = 1220,  $z = -1.8$ ,  $P = 0.06$ ; Fig. 5b) relative to controls.

312 *465 days post-pairing census.* The antibiotic continued to have a  
 long-term negative effect on colony reproduction. All fitness parameters of  
 314 surviving rifampin-fed reproductives were significantly reduced relative to  
 controls (Fig. 5).

316 *730 days post-pairing census.* Two years post-pairing, the negative  
 effect of rifampin on colony fitness persisted despite the antibiotic  
 318 treatment being provided only during the initial stages of colony foundation  
 (Fig. 4). After controlling for the effects of mass (see below), sibship and  
 320 colony of origin, treatment significantly influenced colony fitness ( $t = -2.9$ ,  
 $P = 0.004$  for eggs,  $t = -3.8$ ,  $P < 0.0001$  for larvae and  $t = -4.1$ ,  $P < 0.0001$   
 322 for soldiers; by multivariate linear regression (SPSS, 57). By the last  
 census, 69.8% of the 128 originally established control colonies had  
 324 oviposited at least one egg while only 38.6% of the 120 original rifampin-  
 treated colonies had done so (Pearson's  $X^2 = 24.0$ , d.f. = 1,  $P < 0.0001$ ).

326 Moreover, 63.5% of the original control colonies produced at least one  
 larva whereas only 28.6% of the original rifampin-treated colonies did  
 328 (Pearson's  $X^2 = 30.0$ , d.f. = 1,  $P < 0.0001$ ).

330 **Survival of *Reticulitermes. flavipes* primary reproductives and**  
**colony fitness.** *R. flavipes* reproductives treated with antibiotic had a  
 332 comparable survival rate to the controls for the first 5 months of colony  
 life. A Cox proportional regression indicated that neither colony of origin,



334 sibship, gender nor treatment were significant and independent predictors  
of termite survival (Wald Statistic (WS) = 0.2, 1.5, 0.002 and 0.07, d.f. = 1,  
336  $P > 0.2$ , respectively). In regards to fecundity, after 5 months of colony  
formation 69.4% of the original control established colonies oviposited at  
338 least one egg relative to 53.3% of the original rifampin-treated colonies  
(Pearson's  $X^2 = 2.5$ , d.f.= 1,  $P > 0.05$ ). Approximately 45% and 44% of  
340 the original control and rifampin-treated colonies hatched at least one larva,  
respectively (Pearson's  $X^2 = 0.002$ , d.f.= 1,  $P > 0.05$ ). After 150 days post-  
342 establishment, no soldiers had differentiated. Although these proportions  
were not statistically significant, several additional reproductive parameters  
344 of the rifampin-treated reproductives were negatively impacted relative to  
controls. Rifampin-fed *R. flavipes* reproductives had fewer maximum  
346 number of eggs (MW = 242.5,  $z = -2.7$ ,  $P = 0.007$ ), fewer maximum  
number of larvae (MW = 159.0,  $z = -2.8$ ,  $P = 0.005$ ) as well as fewer  
348 number of larvae on day 150 post-pairing (Mann Whitney U test (MW) =  
112.0,  $z = -3.5$ ,  $P < 0.0001$ ; Fig. 6). Although some additional reproductive  
350 parameters were reduced for rifampin-fed reproductives, they were not  
statistically different. Larger sample sizes and longer surveys past the first  
352 150 days post-establishment are needed to elucidate if rifampin has similar  
long-term effects on *R. flavipes* reproduction as it had in *Z. angusticollis*.

354 Taken together, these results indicate that small amounts of  
rifampin provided during the incipient stages of colony foundation alter the  
356 reproductive output of both termite species for the long term.

358       **Termite mass.** The mass of each surviving *Z. angusticollis* reproductive  
was recorded on day 50 and 465 post-establishment (Table 2). Our results  
360 show that by day 50, control reproductives were no more than 0.005 grams  
heavier than their rifampin-fed counterparts. These differences, although  
362 small, were significant (Table 2). On day 465 post-pairing, the differences  
in mass of the surviving reproductives were reversed and now rifampin-fed  
364 reproductives were heavier than their respective controls (Table 2). The  
reversal in the weight differences from day 50 to day 465 was apparently  
366 due to accelerated weight loss in the controls for both BDTK17 (0.060 g vs.  
0.052 g,  $t = 6.0$ , d.f. = 180,  $P < 0.001$ ) and BDTK19 (0.063 g vs. 0.057 g,  $t$   
368 = 4.1, d.f. = 164,  $P < 0.0001$ ), rather than significant weight gain in the  
antibiotically-treated termites of BDTK17 (0.055 g vs. 0.053 g,  $t = 0.96$ ,  
370 d.f. = 131,  $P = 0.3$ ) and BDTK19 (0.058 g vs. 0.060 g,  $t = -1.6$ , d.f. = 113,  
 $P = 0.1$ ). The significant weight loss of controls could be due to a higher  
372 investment of their energetic reserves in reproduction than that of the  
antibiotic treated reproductives, which consistently had lower reproductive  
374 output.

On day 150 post-pairing, the mass of control and rifampin-treated  
376 *R. flavipes* male and female reproductives were not significantly different  
(males: average  $\pm$  S.D. =  $0.0042 \pm 0.0007$  vs.  $0.0038 \pm 0.0006$  respectively,  
378  $t = 1.9$ , d.f. = 39,  $P > 0.05$ ; females:  $0.0046 \pm 0.0007$  vs.  $0.0042 \pm 0.0007$   
respectively,  $t = 1.7$ , d.f. = 39,  $P > 0.05$ ) and therefore, we conclude that  
380 the addition of rifampin to the diet of *R. flavipes* reproductives did not  
cause malnutrition, starvation or higher mortality relative to controls.

382

## DISCUSSION

384           This investigation demonstrates that the addition of the antibiotic  
rifampin to the diet of *Z. angusticollis* and *R. flavipes* during colony  
386           establishment reduces bacterial diversity in the reproductive's guts, as well  
as colony fitness. Relative to controls, rifampin-treated *Z. angusticollis*  
388           reproductives had reduced survival and lower reproductive success. They  
exhibited a delayed first oviposition and significantly lower production of  
390           eggs, larvae and soldiers throughout the 730 days of colony life (Figs. 4, 5).  
Similarly, rifampin treatment in *R. flavipes* showed a reduction in the total  
392           number of eggs and larvae during the first 150 days of colony foundation  
(Fig. 6). How does rifampin treatment mediate the fitness costs on  
394           reproduction in these termite species? We propose two possible  
explanations.

396           First, the antibiotic could influence reproductive success of  
reproductives indirectly by compromising the nutritional health of the royal  
398           pair, causing reduced weight gain and reproductive output. Rifampin could  
have caused defaunation of the eukaryotic hindgut microbes resulting in  
400           malnutrition and/or starvation. The elimination of wood-digesting  
protozoan symbionts through the use of antibiotics has previously been  
402           demonstrated (11, 26, 53). However, in this study, rifampin-treated termites  
had numerous protozoa (median number =  $7 \times 10^6 \pm 9.3 \times 10^6$  protozoa per  
404           gram of termite) 14 days post-treatment, and it is the gut protozoa that are

primarily responsible for cellulase activity in the digestive tract of primitive  
406 “lower” termites (13, 37). Rifampin does not have a prolonged negative  
effect on the cellulolytic gut protozoa of *Z. angusticollis*, most likely  
408 because this antibiotic specifically inhibits the bacterial RNA polymerase  
(32). Moreover, the most abundant bacteria in the termite’s hindgut, the  
410 spirochetes, play an important role in the digestion process and are highly  
resistant to rifampin (12). Hence, the facts that (i) rifampin did not  
412 eradicate protozoan symbionts of *Z. angusticollis*, (ii) body mass of *Z.*  
*angusticollis* reproductives was transiently affected (Table 2) and was  
414 unaffected in *R. flavipes* and (iii) the experimental replicates survived up to  
the 465 and 730 day (for *Zootermopsis*) and 50 day (for *Reticulitermes*)  
416 census while continuing to show a reproductive output biased against  
rifampin treatment, do not support antibiotic toxicity, malnutrition and/or  
418 starvation as factors reducing fitness. Furthermore, endogenous production  
of cellulases has been reported in this insect order and hence, termite  
420 nutrition may not be completely dependent on their protozoa communities  
(7, 25, 71, 72, 77).

422           Similar studies using antibiotics in the phylogenetically-related  
roach *Periplaneta americana* resulted in poor growth and reduced  
424 reproductive output (54). These effects were attributed to the elimination of  
*Blattabacterium* which mobilizes nitrogen from urate waste deposits within  
426 the fat tissue. They also provide vitamins, proteins and essential amino  
acids to the roach (3, 4, 54, 59). Although *Z. angusticollis* lacks an  
428 association with *Blattabacterium* (59), other bacteria, including the

430 rifampin-eliminated Bacteroidetes and *Treponema*, are similarly involved  
in nitrogen fixation (11, 13, 36, 46) and/or the production of  $\text{NH}_3$  from uric  
acid (52, 59, 66). The absence of these taxonomic groups may have  
432 irreversibly restricted nitrogen availability in female reproductives. Given  
that dietary nitrogen supplementation is known to significantly increase  
434 ovariole number and fecundity in *Z. angusticollis* neotenics and other  
insects (5, 10), the loss of the Bacteroidetes and Mollicutes may have  
436 compromised nitrogen reserves and/or the essential amino acids required  
for oogenesis. However, some Epsilon- and Gammaproteobacteria, two  
438 classes that were overly-represented in the treated guts, may perform  
ammonification, denitrification and nitrogen fixation (38, 46). Thus, further  
440 work is required to associate the fitness cost in treated termites with a shift  
in the ability to use nitrogen.

442           A second possible explanation for the long-term fitness costs  
associated with antibiotic treatment is that rifampin disrupted one or more  
444 mutualistic bacterial partnerships within the termite hosts. Specifically, a  
partnership(s) that goes beyond the breakdown of cellulose. Given the long  
446 co-evolutionary history between the gut symbionts and termites, it is likely  
that these social insects accrue additional benefits from their microbiota that  
448 are unrelated to cellulolytic activity. Microbes can play other important  
roles within their termite hosts including detoxification (17), mediation of  
450 disease resistance and immune function (15, 23, 31, 51, 58, 60, Schultheis  
et al., in preparation), production of volatile compounds that are co-opted  
452 to function as aggregation or kin recognition pheromones and defensive

secretions (2, 24, 28, 39, 45, 47), as well as performing atmospheric  
454 nitrogen fixation (5, 11, 36). Results from this work suggest that the  
microbial communities of *Z. angusticollis* and *R. flavipes* may also  
456 contribute to the fecundity of reproductives and ultimately, to the  
successful establishment of colonies. One such candidate for affecting  
458 reproduction is *Wolbachia pipientis*, a widespread intracellular bacterium  
known to infect *Z. angusticollis* (9). However, based on PCR surveys of the  
460 *Wolbachia wsp* gene from antibiotic-treated and untreated reproductives,  
*Wolbachia* was not involved in influencing colony fitness since all  
462 reproductives, nymphs and eggs from both the experimental and control  
colonies harbored *Wolbachia* regardless of treatment and colony of origin.

464           The bacteria identified in our control animals have previously been  
associated with termite guts, either as normal symbionts (34, 35, 40, 48, 70)  
466 or as opportunistic pathogens (75; Table 1). The long-term fitness costs  
likely resulted from perturbations in the termite gut symbionts in treated  
468 termites. Rifampin is a bactericidal antibiotic that preferentially targets  
gram-positive bacteria (78, 81). The consequence of employing this  
470 antibiotic is that it shifted the gut microbial community largely towards  
gram-negative microorganisms including known termite symbionts;  
472 Termite group 1, *Desulfovibrio* sp., and *Trepanema* sp. (Table 1).

          The most striking change was the abundance of an  
474 Epsilonproteobacterium that was not represented in the control termite  
library. This bacterium's 16S sequence is 98% similar to a rare symbiont of  
476 the termite luminal lining (35) and appears to have increased its

proportional representation in the gut microbiota. At least two potential  
478 reasons for this shift in the dominant bacteria exist. First, the decline in  
gram-positive bacteria may have allowed rare members of the community  
480 such as the gram-negative Epsilonproteobacterium to exploit the new,  
unoccupied niche space of the gut. Members of the rare biosphere  
482 potentially offer an unlimited source of microbial diversity that flourishes  
upon ecological perturbations (64). By altering the normal microbiota of  
484 the gut with antibiotics, rare but relatively fast growing microaerophilic  
species (i.e. some proteobacteria and *Serratia*) not susceptible to the  
486 antibiotic may now exploit the host niche as well as the levels of available  
oxygen, ultimately overgrowing and becoming dominant in the gut (14, 27,  
488 41, 58, 79 and references therein). Second, the appearance of rare or non-  
native bacterial members in the rifampin-treated guts may be impacted by  
490 interactions with other bacteria. For example, the *Serratia marcescens* 16S  
rRNA gene sequence identified in our study is 99.9% identical to an  
492 opportunistic pathogen of termites that has been hypothesized to induce  
replication of normal termite gut bacteria by suppressing the host  
494 immunity, changing available oxygen, and producing bacterial growth  
promoting enzymes like carboxymethylcellulase (1, 19, 68, 75). A  
496 *Serratia*-induced proliferation of the symbiotic community could cause  
septicemia that can result in early termite mortality (75). *S. marcescens* is  
498 present in the rifampin gut library, but not in the control termite gut library.  
Thus, its appearance in the treated termites may have directly or indirectly  
500 led to the proliferation of the rare Epsilonproteobacterium symbiont of the

luminal lining (48). Yet, it is important to keep in mind that not all  
502 associations with *Serratia* are necessarily pathogenic. *Serratia grimesii*, for  
example, has been implicated as a source of folate compounds important to  
504 the maintenance of a functional hindgut microbiota of *Z. angusticollis* (29).  
The shift in gut bacterial population structure is strikingly prolonged since  
506 termites were not fed antibiotics for ~50 days prior to dissections. The  
inability to return to a pre-treatment microbial homeostasis (70), coupled  
508 with the acquisition of putative, opportunistic pathogens and the slow  
growth rates of many of these termite gut microorganisms (27, 30, 41, 42,  
510 62), may help explain the prolonged effects that the antibiotic had on  
longevity and fecundity.

512           This study provides the first report of the long-term fitness  
consequences of disrupting the normal gut microbiota of termites. The long  
514 coevolutionary history of termites and their associated microbiota, coupled  
with the environmentally stable conditions inside their nests, lends itself to  
516 study the nature and dynamics of symbiotic interactions (33). The  
mutualistic gut partnerships of social insects may impact not only the  
518 fitness of individuals but also have significant repercussions at the colony  
level. Symbionts, whether parasitic, commensal or mutualistic, pose  
520 important selective pressures on their hosts. These host-microbial  
interactions likely influence the evolution of multiple host life history traits  
522 including longevity, behavior, reproductive biology, immunity and the  
evolution and maintenance of sociality (20, 33, 56, 61, 69,74 and  
524 references therein). Furthermore, the use of rifampin and/or other



antibiotics has potential applicability for biological control of social insect  
526 pests. By disrupting the mutualistic interaction between termite hosts and  
their symbionts, better management practices of these social insect pests  
528 may be achieved without the environmental and ecological drawbacks  
typically associated with the use of other toxic chemicals.  
530

532

## ACKNOWLEDGMENTS

534

536           We thank the administrators of Huddart Park for allowing collection  
of termite colonies as well as Jessica Dumas, Larissa Gokool, Zea Schultz,  
Patrick Henrick, Brian Lejeune and Troy Kieran for help performing  
538 census and establishing colonies. We also appreciate the helpful comments  
and suggestions of two anonymous reviewers

540

This research was funded by the Louis Stokes Minority Program which  
supported Jessica Dumas, NSF CAREER award DEB 0447316 to  
542 Rosengaus RB, and NSF IOS-0852344 and NAI NNA04CC04A to  
Bordenstein SR.

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## FIGURE LEGENDS

FIG. 1. Diagrammatic representation of the protocol. Incipient colonies of *Z. angusticollis* were established by paring dealates inside Petri dishes lined with filter paper and wood on day zero. Arrows indicate the days at which rifampin was added to the experimental colonies. Control colonies received distilled water only on these same days. In subsequent census, colonies were sprayed with distilled water as needed. Pd indicates that incipient colonies were housed in Petri dishes. Q and K denote queen and king, respectively. See text for details.

FIG. 2. Survival distributions of control (solid line) and rifampin-treated (dashed line) male and female *Z. angusticollis* reproductives originating from colony BDTK17 (a) and BDTK19 (b) during the first two years of colony life. Filled and open circles represent the percent survival of control and rifampin-treated individuals at each of the major census dates (indicated by the arrows), respectively. These percentages differed significantly on days 465 and 730 post-establishment (Pearson's  $X^2$ ,  $P < 0.004$ ). \* and NS above the arrows represent significant and insignificant differences in the median survival time at each of the census dates, respectively (MW test; see text). Additional survival parameters are shown in Table 2.

832

FIG.3. Percent number of established control ( □ ) and rifampin-treated ( ▒ ) colonies in relation to the number of eggs (a), larvae (b) and soldiers (c) produced 150 days post-establishment. Kolmogorov-Smirnov tests and their associated Z score were used to test for differences in the location and shape of the distributions and whether the two treatments had equal distributions. Note that a higher percentage of control colonies produced the highest number of eggs, larvae and soldiers.

840

FIG. 4. Number of eggs (a), larvae (b) and soldiers (c) produced by control ( □ ) and rifampin-treated ( ▒ ) *Z. angusticollis* reproductives at each of the major census days. Each boxplot shows the median value and interquartile range. The outliers, identified by small circles, included cases with values between 1.5 and 3 box lengths from the upper edge of the box. The numbers below each of the boxplots represents the number of colonies. Reproductive parameters between treatments within each census day were compared by MW test.

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FIG. 5. Number of days elapsed to first oviposition (a) and first hatching (b) for *Z. angusticollis* colonies headed by untreated ( □ ) and rifampin-treated reproductives ( ▒ ). Each boxplot shows the median value and interquartile range. The outliers, identified by small circles, included cases with values between 1.5 and 3 box lengths from the upper edge of the box.

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Reproductive parameters between treatments were compared using MW  
test.

FIG. 6. Maximum number of eggs, maximum number of larvae and  
number of larvae recorded on day 150 post-colony establishment produced  
by control (□) and rifampin-treated (▣) *R. flavipes* reproductives. Each  
boxplot shows the median value and interquartile range. The numbers  
below each of the boxplots represents the number of colonies. Numbers  
below each of the boxplots represents the number of colonies.  
Reproductive parameters between treatments were compared using a non-  
parametric Mann-Whitney U test.

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TABLE 1. Number of 16S rRNA OTUs in *Zootermopsis* control and treated guts. Refer to Fig S1 in supplemental material to see corresponding rarefaction curve.

Class	Bacteria genus	Control Gut	Refampacin Gut	References
Endomicrobia	Termight Group 1	19	0	Hongoh et al., (2003); Nakajima et al., (2005); Kudo (2009)
Bacteroidetes	Bacteroides	22	7	Nakajima et al., (2005); Kudo (2009)
Betaproteobacteria	Propionibacter	1	4	
Deferribacteres	Lincoln Park 3'	1	0	Kudo (2009)
Acintobacteria	Treponema	19	0	Kudo (2009)
Verrucomicrobia	Verrucomicrobia	6	0	Hongoh et al., (2005)
Clostridia	Clostridiales	7	0	Hongoh et al., (2005); Nakajima et al., (2005); Kudo (2009)
Clostridia	Uncult Rumen bacterium (<95%)	2	0	Hongoh et al., (2005); Nakajima et al., (2005); Kudo (2009)
Betaproteobacteria	Uncult Sludge (<95%)	2	0	
Verrucomicrobia	Opitutaceae	1	0	Hongoh et al., (2005); Nakajima et al., (2005); Kudo (2009)
Epsilonproteobacteria	Sulfurospirillum	1	0	Hongoh et al., (2003)
Betaproteobacteria	Uncult Beta-proteo	3	0	
Gammaproteobacteria	Uncult Gamma-proteo (<95%)	1	0	
Deltaproteobacteria	Uncult Desulfovibrionales (<95%)	3	0	Hongoh et al., (2005); Nakajima et al., (2005); Kudo (2009)
Gammaproteobacteria	Pseudomonas	0	13	Veivers et al., (1982); Devi and Kothamasi (2009)
Bacilli	Enterococcus	0	3	
Gammaproteobacteria	Providencia	0	1	Thongaram et al., (2005)
Betaproteobacteria	Oxalobacteraceae	0	1	Veivers et al., (1982)
Alphaproteobacteria	Methylobacterium	0	11	
Alphaproteobacteria	Afipia	0	20	
Acintobacteria	Arthrobacter	0	13	Hongoh et al., (2003); Kudo (2009)
Flavobacteriales	Cytophaga	0	4	
Betaproteobacteria	Ralstonia	0	4	

Bacteroidetes	Uncult bacterium (<95%)	3	2	Nakajima et al., (2005); Kudo (2009)
Bacilli	Streptococcus	0	1	Thongaram et al., (2005)
Alphaproteobacteria	Sphingomonas	0	2	
<b>Total number of clones</b>		<b>91</b>	<b>86</b>	

TABLE 2. Survival parameters and mass estimates of control and rifampin-treated *Z. angusticollis* primary

882                      reproductives originating from two parental colonies across the first two years post-establishment.

	BDTK17			BDTK19		
	Control	Rifampin	$P^{\dagger}$	Control	Rifampin	$P^{\dagger}$
<b>LT<sub>50</sub> on day 730</b>	730 ± 31	465 ± 39	$P < 0.0001$ BS = 1 8.6	730 ± 29	465 ± 46	$P = 0.008$ BS = 7.0
<b>% survival on day 730</b>	26.8	4.1		27.3	11.1	
<b>Hazard ratio of death</b>	Reference	1.7X	$P < 0.0001$ WS = 19.0 d.f. = 1	Reference	1.4 X	$P = 0.07$ WS = 5.5 d.f. = 1
<b>Mass (in grams) on day 50 post-pairing</b>	0.0604 ± 0.01 ( <i>N</i> = 100)	0.0549 ± 0.009 ( <i>N</i> = 92)	$t = 3.8$ d.f. = 190 $P^{\S} < 0.0001$	0.0626 ± 0.008 ( <i>N</i> = 95)	0.0577 ± 0.009 ( <i>N</i> = 74)	$t = 3.6$ d.f. = 167 $P^{\S} < 0.0001$
<b>Mass (in grams) on day 465 post-pairing</b>	0.0518 ± 0.008 ( <i>N</i> = 79)	0.0530 ± 0.01 ( <i>N</i> = 40)	$t = -0.7$ d.f. = 117 $P^{\S} = 0.4$	0.0567 ± 0.009 ( <i>N</i> = 71)	0.060 ± 0.009 ( <i>N</i> = 41)	$t = -2.0$ d.f. = 110 $P^{\S} = 0.04$

884                       $^{\dagger}$  indicate differences in the survival distributions between control and rifampin treated

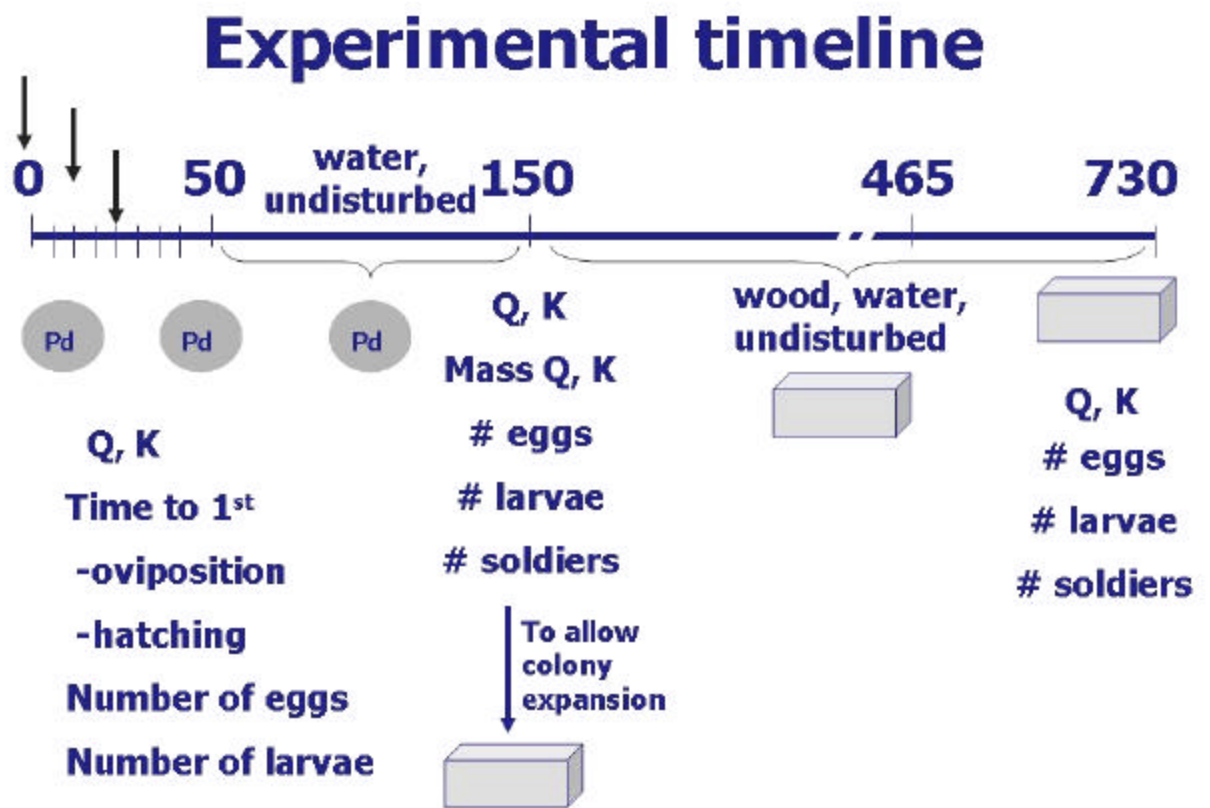
reproductives. These distributions are depicted in Figs. 2a,b. BS=Breslow statistic (Survival

886                      analysis), WS=Wald Statistic (Cox proportional regression).  $^{\S}$  denotes differences in the average



mass between control and rifampin treated reproductives within each parental stock colony (*t* test,  
SPSS). The numbers in parentheses indicate the number of surviving individuals on which mass  
averages were based upon.

Fig. 1.



906 Fig. 2.

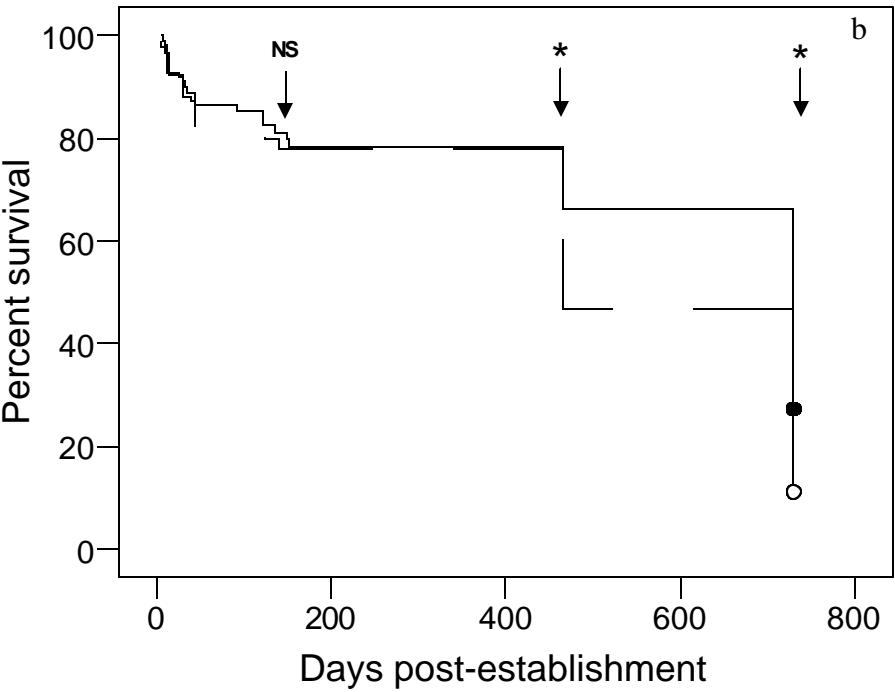
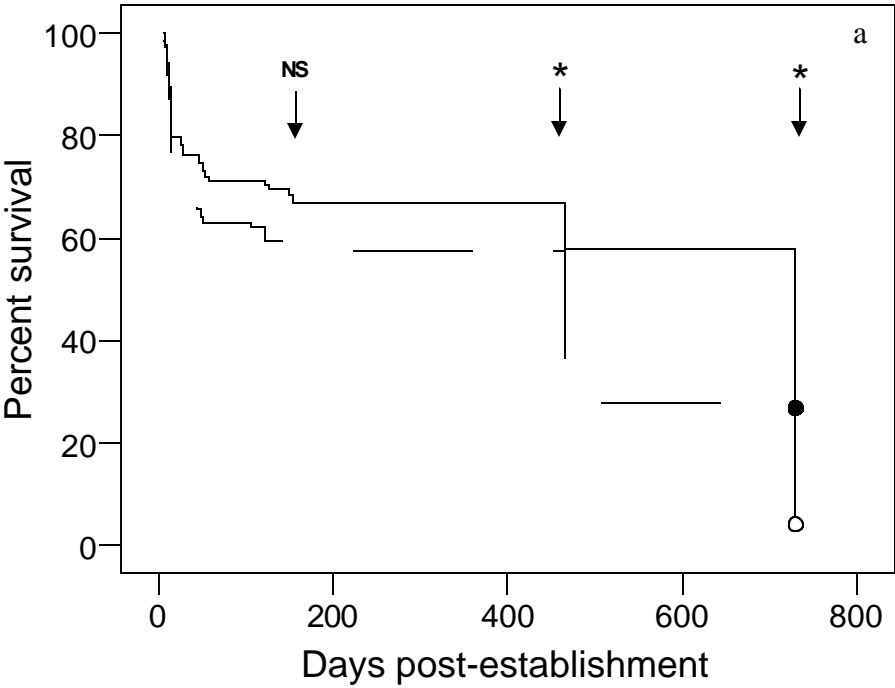


Fig. 3.

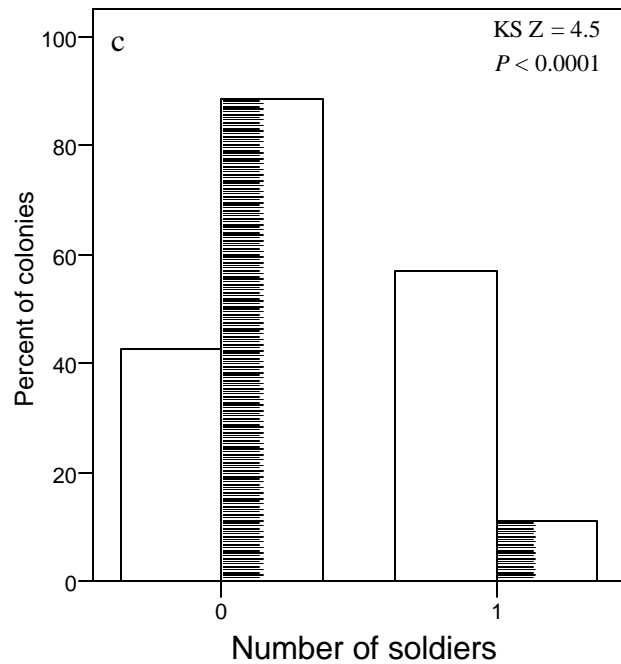
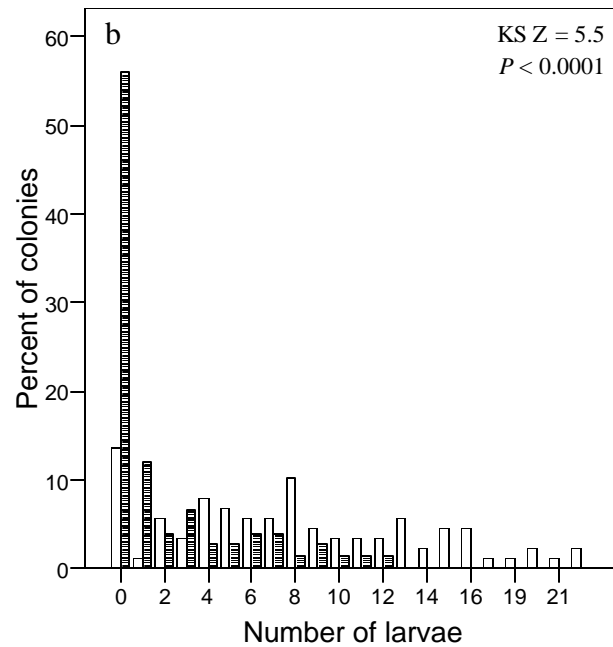
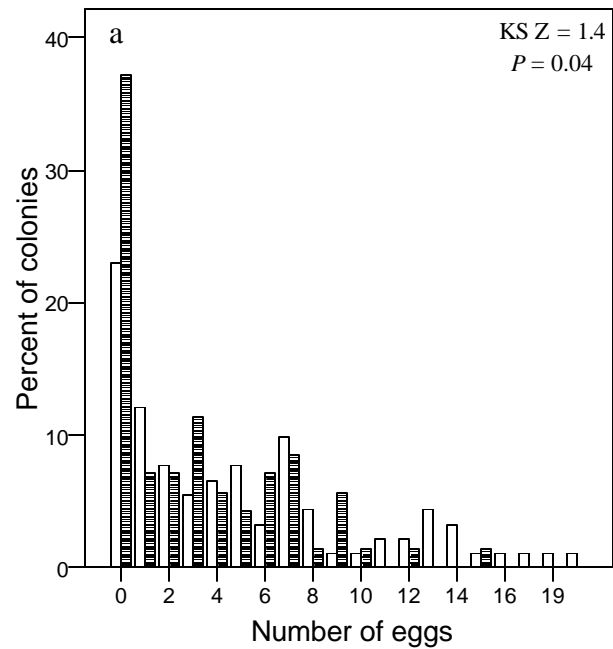


Fig. 4

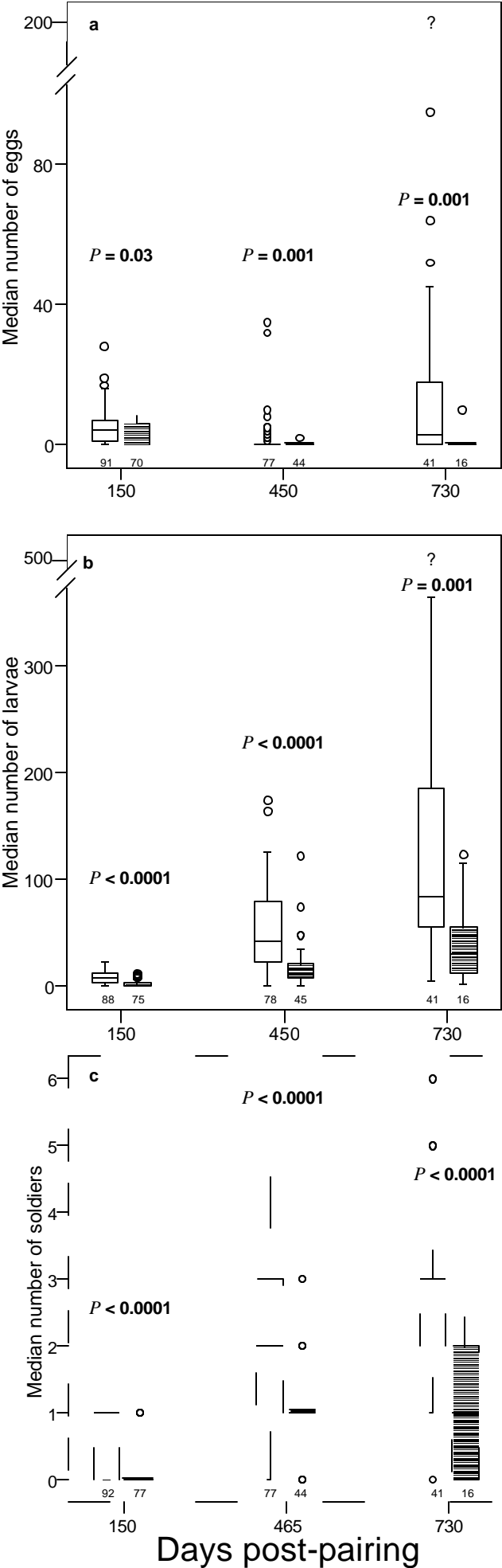
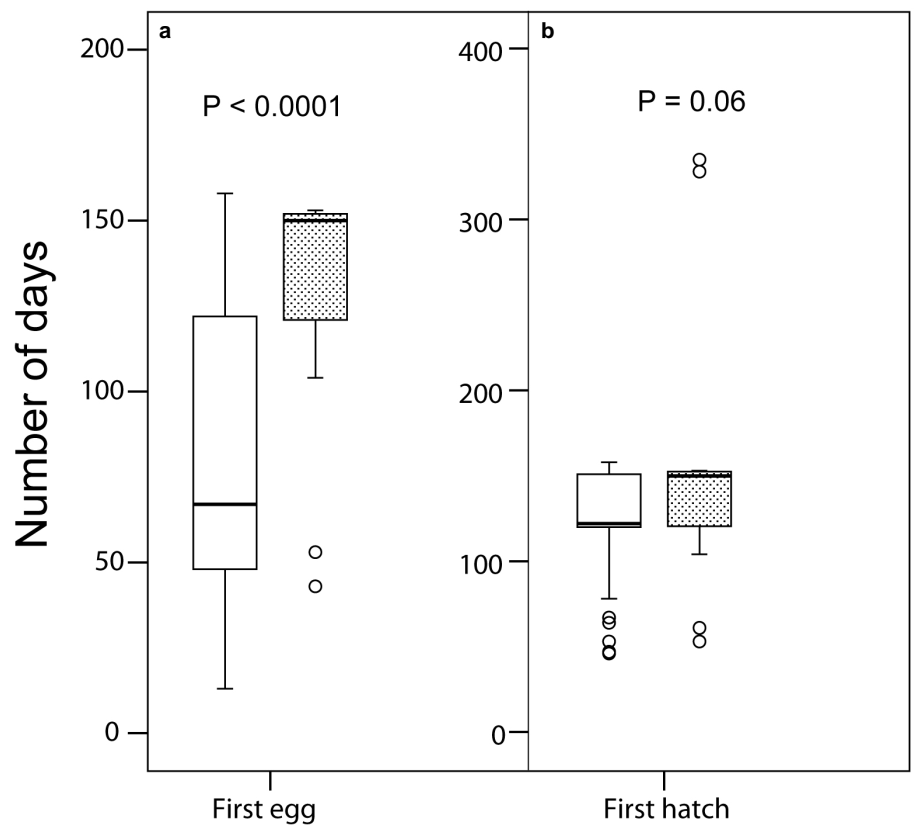
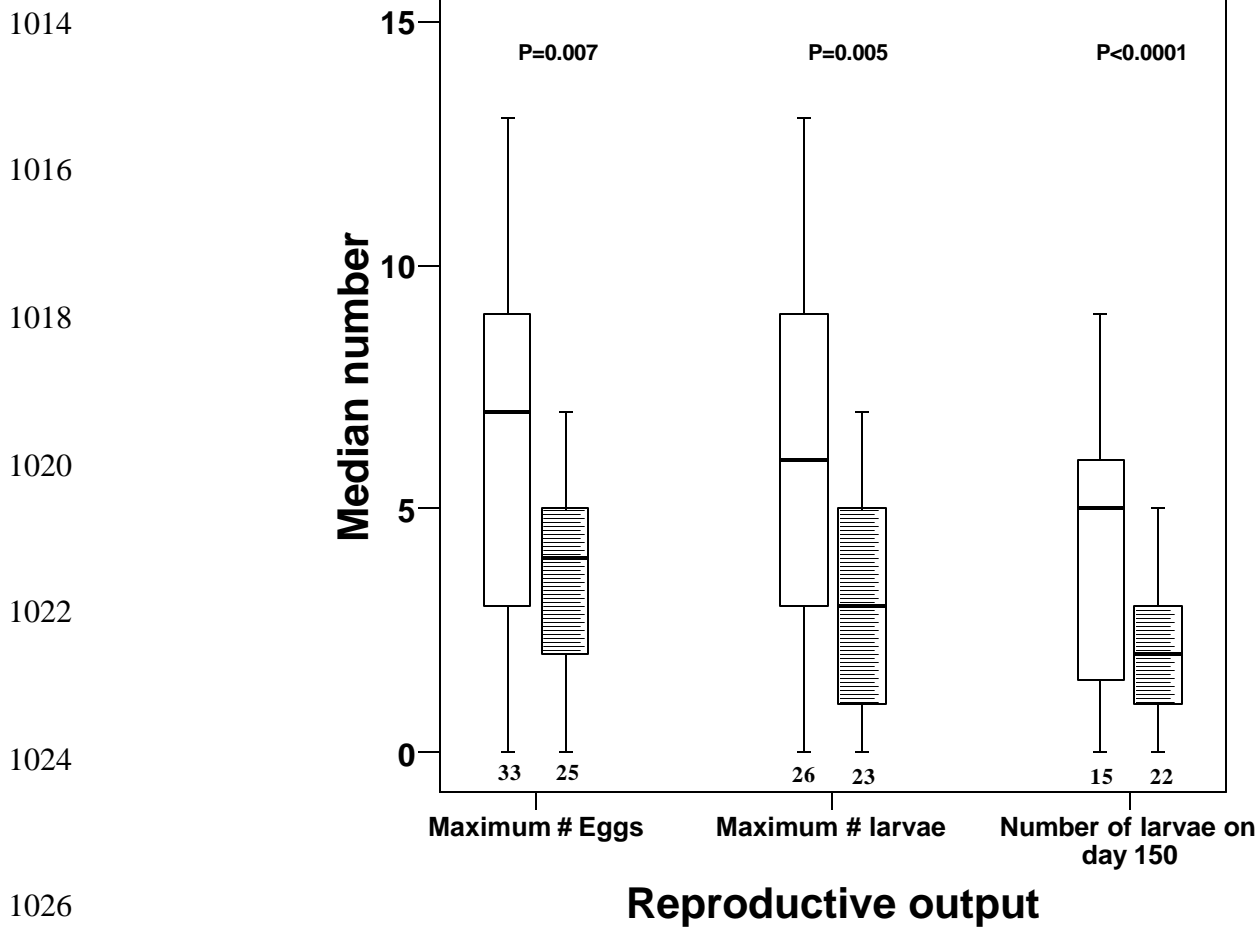


Fig. 5.



1012 Figure 6



## Supplemental Material

### Disruption of termite gut-microbiota and its prolonged fitness consequences

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## METHODS

**PCR, Cloning, and Sequencing.** To prepare samples for cloning, PCR amplification of the bacterial 16S rRNA gene was performed using 5 µl of the DNA samples as template. This template was combined into a 50 µl reaction using 15.8 µl of H<sub>2</sub>O, 10 µl of 5x Buffer (Promega, Madison WI), 5 µl of 2.5 mM dNTP's (Invitrogen, Carlsbad, CA), 6 µl of 25mM MgCl<sub>2</sub> (Promega), 0.2 µl GoTaq<sup>®</sup> Flexi (Promega), 4 µl of 5uM forward primer 27F (5'-AGAGTTTGATCMTGGCTCAG-3', Sigma-Aldrich), and 4 µl of 5uM reverse primer 1492R (5'-ACGGCTACCTTGTTACGACTT-3', Sigma-Aldrich; Suzuki and Giovannoni, 1996). The thermalcycling program was set up as follows: 94°C (5 min), then 35°C repeats of 94°C (1 min) 55°C (45 sec) 72°C (2 min), followed by 72°C (15 min). 5 µl of the resulting amplicon products were run on a 1% agarose gel (Fisher Scientific), stained with the nucleic acid stain GelRed (Biotium, Hayward, CA), and imaged for the proper band size under UV illumination. The remaining amplicon product is run on a 1% low melting point agarose gel (USB Scientific, Cleveland, OH) and similarly stained. A Dark Reader<sup>®</sup> transilluminator (Clare Chemical Research) was used to image the gel and excise bands for purification with the Wizard<sup>®</sup> SV Gel and PCR



Product Purification Kit (Promega). The resulting gel-purified product was then used for  
1052 cloning and sequencing. The amplicon product was ligated using the Invitrogen Topo TA  
cloning Kit for Sequencing (4-TOPO V2, vector) and the resulting vector was used to  
1054 transform One Shot® Top 10 Chemically Competent transformation cells (Invitrogen),  
per the manufacture's recommended procedure. Plasmid inserts were unidirectionally  
1056 sequenced at Genewiz® (South Plainfield, NJ) using rolling circle amplification off the  
TOPO vector with inserted amplicon. For the rifampin and control groups, 94 clones were  
1058 sequenced.

**Clone Library and Sequence Processing.** Sequences were trimmed and sorted in  
1060 Geneious® v4.8. Sequences were then aligned using the GreenGenes online workbench  
1062 (<http://greengenes.lbl.gov>) using a 97% identity cutoff, DeSantis *et al.*, 2006). The  
resulting alignment was used to remove any chimeric sequences with the Bellerophon  
1064 Chimera Checker (Ribosomal Database Project v10). Genera and class were assigned to  
each sequence using the GreenGenes Comparison algorithm that searches across three  
1066 nucleic databases (NCBI, RDB, and Hugenholtz). To compare the two libraries (rifampin  
and untreated control) in UniFrac, a representative sequence for each genera in each  
1068 sample type was aligned using a MUSCLE alignment (500 iterations) with all gaps  
removed. A PhyML tree was then generated with a Jukes-Cantor substitution model and  
1070 branch lengths calculated (Guindon and Gascuel, 2003). In total, 171 high-quality, non-  
chimeric, bacterial ribosomal sequences were obtained from the two libraries. The control  
1072 group had a total of 86 assignable sequences and the rifampin group had a total of 85  
sequences, with an average sequence length of 802bp for each library.

## 1074 Statistics

**(i) Effects of antibiotic ingestion on termite gut microbiota.** Rarefaction analysis on the gut microbiota was conducted using the Analytic Rarefaction v2.0 software distributed by Hunt Mountain Software. UniFrac was used to test for differences between clone libraries with 100 permutations. To estimate the microbial diversity, species richness and diversity indices were calculated using Estimates 8.2 (50 runs, randomized with replacement, using the classic Chao1, ACE, and Simpson's reciprocal formulas; Colwell *et al.*, 2008). To determine if occurrences of shared outstanding taxonomic units (OTUs) were significantly different between the two treatments, a Fisher's exact test was conducted. The median number of cultured gut bacteria and protozoa between control and experimental animals were analyzed with MW. Because mass of the reproductives was normally distributed, differences between treatments were analyzed with t-tests.

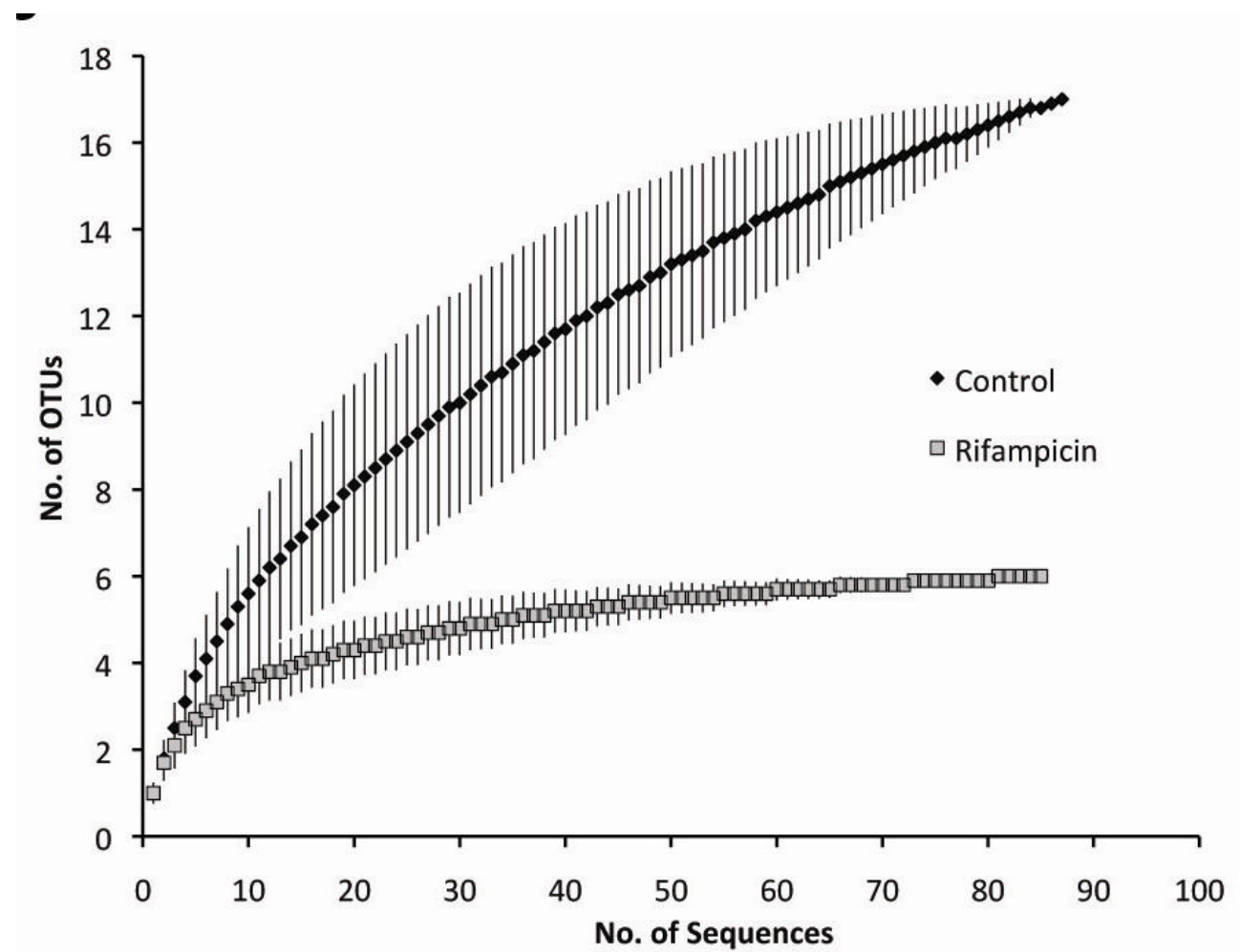
**(ii) Survival of reproductives and colony fitness.** Because all colonies were not established or did not undergo a census on the same day, results were standardized by analyzing survival and reproduction data based on the time elapsed between pairing and each of the subsequent censuses. Survival data was analyzed using both a Cox proportional regression and Survival analyses (SPSS, 1990). While the former analysis helps identify which variables are significant and independent predictors of death, the latter allows the estimation of several parameters including the number of days elapsed since pairing until 50% of the individuals died (median survival time; LT<sub>50</sub>), percent survival at the end of the census period and the time course of survival (or survival distributions). In addition, we calculated the likelihood with which animals in the experimental treatment died relative to control termites (or relative hazard ratios of death). These hazard functions therefore, characterize the instantaneous rate of death at a particular time, given that the individual survived up to that point, while controlling for

the effect of other significant variables on survival (Cox regression model; SPSS, 1990;  
1102 Rosengaus *et al.*, 2000).

Reproductive output was analyzed by comparing the frequencies of distributions  
1104 with which eggs, larvae and soldiers were produced by the control and rifampin treated  
reproductives on day 150 post-pairing [Kolmogorov-Smirnov Z test (KS), SPSS, 1990].  
1106 In addition, differences in the number of colonies with eggs, larvae and soldiers as a  
function of treatment two years post-pairing were analyzed with 2x2 Pearson's  $X^2$  tests.  
1108 The number of offspring produced by 50% of the colonies in each of the control and  
rifampin treatments (median number of eggs, larvae and soldiers) was compared for each  
1110 of the census dates with non parametric Mann-Whitney U tests (MW), as well as  
differences in the number of days elapsed since pairing until 50% of the  
1112 colonies/treatment oviposited their first egg and hatched their first larva.

**FIGURE LEGEND**

**Fig. S1 Supplemental .** Rarefaction analysis of OTUs from control and rifampin-treated hindguts. The arc of the curve indicates the likelihood of sequencing a new bacterial OTU if more clones were sampled. The curve is generated based on the occurrences of OTUs within the clone libraries.



**Supplemental TABLE 1.** 16S rRNA gene homology of *Zootermopsis* gut bacteria to

species with known mutations for rifampicin resistance

Species with rifampin resistance gene			
Bacterial genus in termite gut	Bacterial OTU with known rifampin resistance	NCBI GI	16S % Pairwise id
Arthrobacter	Arthrobacter sp. FB24	116668568	95.0%
	Arthrobacter aureus TC1	119960487	92.7%
	Arthrobacter arilaitensis Re117	308175814	92.7%
Cytophaga	Cytophaga hutchinsonii ATCC 33406	110279108	81.4%
Enterococcus	Enterococcus faecium strain DSM 10663	42560437	96.9%
Pseudomonas	Pseudomonas fluorescens SBW25	229359445	96.3%
	Pseudomonas aeruginosa strain MYL-21	321159400	94.5%
Treponema	Treponema sp. ZAS-1	4235383	97.8%
	Treponema phagedenis strain YG3903R	219551879	91.7%
	Treponema medium strain G7201	310975273	91.4%
	Treponema socranskii subsp. Socranskii	2653628	90.6%
	Treponema denticola ATCC 35405	41821838	91.3%
Verrucomicrobium	Treponema pallidum	176249	90.1%
	Verrucomicrobium spinosum DSM 4136	219846674	78.3%

An analysis was conducted to determine whether the frequency of rifampin-resistant bacteria are more frequent post-treatment vs. pre-treatment, as would be expected if treatment selected for rifampin resistant bacteria. The results indicate that there are relatively few OTUs observed in the termite gut (before or after rifampin treatment) that are closely related to known rifampin resistant bacteria. Further, if we assume that if a strain in our dataset is related to a resistant strain, then they themselves are resistant, we still observe that the frequency of strains with rifampin resistance is low and the same between pre- and post-treatments ( $P = 0.656$ , Fisher's exact test). Thus, we have not selected for rifampin resistance by treating the termites with antibiotic. OTU denoted operational taxonomic unit.

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